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(54) Title: SEPARATION OF AMINO ACIDS AND PEPTIDES FROM PROTEIN HYDROLYSATES

(57) Abstract

A method for the separation of hydrophobic amino acids and peptides containing aromatic groups from protein hydrolysates by the use of anion exchange media at substantially neutral pH and the regeneration of the media for further use.

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Separation of amino acids and peptides from protein hydrolysates

The invention relates to a method of separating amino acids and peptides from protein hydrolysates. In particular the invention relates to the removal of hydrophobic amino acids and peptides containing aromatic groups from protein hydrolysates by the use of anion exchange media thus improving the taste quality of the hydrolysate.

Background Art

Hydrolysates are produced by enzymatic cleavage of proteins to manufacture products with lowered allergenicity and improved nutritional availability (for example hydrolysates of casein, whey and soy proteins are produced commercially). Such hydrolysates are desirable products for use with both humans and animals. In particular, such hydrolysates are useful when the subject has digestive problems such as an incapability to break down whole proteins in the digestive tract. However the action of the enzymes during the hydrolysis process causes the release of hydrophobic peptides which are perceived as bitter. As a result, when used in food products, the maximum level of incorporation of such hydrolysates is limited by the bitter taste. Therefore for oral administration, it is desirable that the amount of these compounds in the product be lowered to achieve an acceptable taste.

It is known that bitter hydrophobic peptides can be lowered in concentration or removed by the use of adsorbents such as activated carbon, or synthetic resins such as XAD4 and XAD7 (Rohm and Haas) or HP20 and HP21 (Mitsubishi Kasei Corporation) where the base matrix is composed of a polymer with hydrophobic properties such as styrene-divinyl benzene, acrylate or methacrylate. Indeed a number of studies and much research has been directed to

the debittering of protein hydrolysates using hydrophobic adsorbents. United States Patent No 4.075,195 to John F Roland entitled "Debittered Protein Product and Method of Manufacture" discloses a process for the removal of the bitter fraction of a protein hydrolysate by passing the hydrolysate through a bed of phenolic resin particles. United States Patent No 5,266,685 to John T Garbutt entitled "Non-Bitter Protein Hydrolysates" discloses the treatment of a protein hydrolysate with a hydrophobic adsorptive resin which is functional to remove the protein hydrolysate bitter taste components. In this US patent there is also disclosure of the perceived undesirability of the use of ion-exchange resins for the debittering process. The use of adsorbents does, however, have several disadvantages in commercial production. The synthetic polymer adsorbents, with the exception of XAD16 (Rohm and Haas) or equivalents, are not currently permitted by the Food and Drug Administration (USA) for use in food applications. In addition, the hydrophobic adsorbents are difficult to regenerate. They require excess alkali and possibly solvents during the regeneration thus adding to the cost of the process.

Japanese Patent 59-159792 to Meiji Confectionary KK entitled "Manufacture of Casein Phosphopeptide" is directed to a new method of manufacture of Casein Phosphopeptide via the hydrolysis of casein by trypsin. The bitter taste, which is caused by the presence of peptides in the trypsin hydrolysate is removed by the use of activated carbon or cation exchange resins. Anion exchange resins are disclosed as being unsuitable for use in the removal of the bitter peptides from the mixture. The patent discloses the reclaiming of the column through the suitable treatment with acid/alkali or by calcination regeneration. This Japanese patent therefore discloses the use of cation exchange resins in connection with the removal of the bitter taste associated solely with trypsin hydrolysates. The disclosure does not teach the applicability of anion-exchange media in connection with the

removal of specific amino acids and peptides from a mixture of protein hydrolysates and also does not teach how regeneration of the anion-exchange media can be achieved. No further reference to this regeneration process is made.

It is therefore an object of an invention to provide an improved method for separating hydrophobic amino acids and peptides containing aromatic groups from protein hydrolysates or at least to provide a viable alternative.

Statement of the Invention

The invention in a first aspect comprises a method for separating hydrophobic amino acids and peptides containing aromatic groups from protein hydrolysates using anion exchange media.

The invention further comprises a method for separating hydrophobic amino acids and peptides containing aromatic groups from a mixture of protein hydrolysates comprising:

(a) using an anion exchange resin at a substantially neutral pH, and thus in a neutral form, to bind and remove said amino acids and peptides from the mixture of protein hydrolysates; and

(b) removing the bound amino acids and peptides from the anion exchange resin by lowering the pH to convert the neutral resin to the ionised form.

Preferably the hydrophobic peptides separated from the protein hydrolysates contain one or more amino acid unit(s) with an aromatic group.

Preferably the anion exchange resin is a hydrophobic base matrix with a weak base ion-exchange functionality.

Preferably the weak base ion-exchange functionality has a pK_a or pK_a's in the pH range of substantially 2.0 to substantially 9.0, more preferably between 2.0 and 6.0.

Preferably the ion-exchange resin is regenerated by removing the bound amino acids and peptides from the ion exchange resin using a combination of low pH and high temperature.

Preferably the pH at which the resin is used to bind the amino acids and peptides is greater than the pH required to completely ionise the weak base functionality. Preferably this pH is between substantially 4.0 to substantially 9.0 and more preferably between 6.5 and 8.0.

Preferably the pH used to remove the bound compounds from the ion exchange resin is sufficiently low to create an ionised form of the weak base functionality. Preferably this is between substantially 1.5 and substantially 6.0 and more preferably between substantially 1.5 and substantially 4.0.

Preferably the temperature used in the regeneration of the ion exchange resin is between substantially 45°C and 100°C, more preferably between substantially 50°C and substantially 100°C, and most preferably between substantially 60°C and substantially 80°C.

Preferably the protein hydrolysates are derived from animal products or plants.

Preferably the protein hydrolysates are hydrolysates of casein, whey, or soy protein.

The invention further comprises a process for the separation of hydrophobic amino acids and proteins containing aromatic groups from protein hydrolysates using a hydrophobic anion exchange resin with a weak base functionality comprising the steps of:

(a) equilibrating or regenerating the resin;

(b) adding the protein hydrolysate to the resin at a pH of between substantially 6.5 to substantially 8.0, at a temperature of between substantially 5°C and substantially 20°C, and at a concentration of between substantially 2% to substantially 20% w/v;

(c) mixing the hydrolysate and the resin;

(d) separating the non-bound product from the resin and rinsing with water; and

(e) regenerating the resin with hot water at a temperature of between substantially 60°C and substantially 80°C and at a pH of between substantially 1.5 and substantially 4.0.

Drawings

The attached Figures show the results of some of the Example processes in graphical form.

In the Figures:

Fig. 1 shows the results of Example 1;

Fig. 2 shows the results of Example 2;

Fig. 3 shows the results of Example 4;

Fig. 4 shows the results of Example 6; and

Fig. 5 shows the results of Example 7.

Detailed Description of the Invention

The invention is directed to the separation of hydrophobic amino acids and peptides containing aromatic groups from protein hydrolysate mixtures using anion exchange media. In the past, ion exchange resins generally have not been thought to be suitable for use in large scale separation processes for this purpose as it was thought that sufficient selectivity of binding the compounds of concern to the surface of the resins did not exist for practical industrial production purposes.

It has now been found that the use of anion exchangers with a weak base functionality and hydrophobic base matrix at a substantially neutral pH will bind hydrophobic amino acids and peptides containing aromatic groups to the surface of the resin and thus remove them from the hydrolysate mix (an adsorption stage).

The types of amino acids and peptides that can be selectively removed are in general terms the hydrophobic amino acids and peptides containing aromatic groups. Such compounds include phenylalanine, tyrosine, tryptophan and peptides thereof as well as others as are well known in the art. When used in the dairy industry, this separation method can be advantageously used to de-bitter hydrolysates of casein or whey for example. The bitter taste caused by combinations of amino acids having hydrophobic end groups can be removed if such compounds are separated relatively selectively from the protein hydrolysates. The use of these hydrolysates in food products, if the bitter taste is reduced, will therefore be greatly enhanced. The process of the invention will not remove all the hydrophobic amino acids/peptides however, as only those containing aromatic groups are preferentially removed. Hydrophobic amino acids/peptides containing only straight chain molecules are only removed

to a very limited extent (see Example 7). Hydrophobic peptides which contain units having aliphatic straight chains will be removed from the hydrolysate provided the peptide also contains units having aromatic groups. The specificity is directed to amino acid units containing aromatic groups and therefore if these are connected to amino acid units containing aliphatic straight chains, the resultant peptide will also be removed from the hydrolysate mixture. This can clearly be seen in Example 7.

The surveyed and selected preferred anion exchange resins will have

(1) hydrophobic base matrices;

(2) sufficiently high surface area available for sufficient binding of the amino acids and peptides;

(3) charge purity - no quaternary charged groups associated with the resin, that, at neutral pH, would be carrying a charge which would interfere with the hydrophobic interaction. This may be compared to most commercial weak anion exchange resins which have some quaternary charge associated with them as a side product of manufacture; and

(4) charge density - sufficient density of chargeable groups on the surface of the resin to be effective in the regeneration of the resin on the lowering of the pH.

The ion-exchangers useful in the process of the invention are generally comprised of a weak base group such as a primary, secondary or tertiary amine with a pK_a or pK_a's in the range of pH 2.0 to pH 9.0 that are attached to a particulate matrix with hydrophobic character such as styrene divinyl benzene. Alternative combinations of weak base groups and particulate matrices as will be known in the art may also be used.

Anion exchange resins are subtantially neutral in charge when the pH of the equilibrating solution is above the pK_a of the base ligand. The preferred anion exchange resins are those with weak base ion-exchange groups that are substantially neutral at neutral pH. The more the equilibrating pH is above the pK_a of the weak anion exchange resin, the lower the density of charged groups on the surface of the resin.

Therefore at neutral pH, the preferred resins for this invention are substantially neutral, enhancing the interaction between the hydrophobic amino acids and peptides and the resin. The pH range can be as wide as pH 4.0 to pH 9.0 depending on requirements as will be known in the art. More neutral pH's between substantially 6.5 and 8.0 are however preferred.

The most suitable resins will have all the above properties. Exemplary commercial resins include: Relite A329 from Sybron/Relite, which is the best of the surveyed resins. Others, such as Purolite A103, Purolite A100 (Purolite International), Amberlite IRA93SP (Rohm and Haas), Dowex MWA1 (Dow Chemical Company) are also suitable, but are not as effective as Relite A329 because their binding capacities are less. Other suitable resins as will be known in the art may also be used.

The process of the invention includes the ability to regenerate the anion exchange resin. This relies on the ability to alter the surface charge of the resin, and thus the hydrophobicity and strength of adsorption of the hydrophobic substances onto the resin. This is achieved by lowering the pH of the solution to substantially lower than the pK_a of the ion-exchange groups on the resin. This induces charged groups on the resin surface thus releasing the bound substances.

The resin used in the process of the present invention can therefore be regenerated by lowering the pH surrounding the resin, preferably at high temperature, causing the amino acids and peptides to unbind from the resin surface thus regenerating the resin for further use (a regeneration step). The pH can be lowered by the use of any suitable acid or buffer (eg HCl, H_2SO_4 etc) as will be known in the art. Preferably the pH is lowered to between substantially 1.5 to 6.0 although a pH between 1.5 and 4.0 is considered most suitable. Further a temperature of between 45°C and 100°C enhances the regeneration step. This may be achieved by the use of hot water or the like as will again be known in the art. Temperatures between 50°C and 100°C are preferred with temperatures between 60°C and 80°C being considered most suitable. The hydrophobic ion-exchangers with weak base functionality have been substituted for conventional non-ion exchange adsorbents with the amino acids binding to the ion-exchanger in the uncharged form (ie at the substantially neutral pH). A shift to lower pH to convert the ion-exchanger to the charged form results in the release of the bound amino acids.

It is thought that the acid used in the regeneration step also neutralises the carboxyl groups of the amino acids and peptides giving them a nett positive charge which is repelled by the nett positive charge of the ion exchange resin. In this way the process of the present invention can be carried out with resins that have an established history and acceptability of use in food processes. Moreover, the resins can be readily regenerated for reuse without the application of strong caustic, acids or toxic solvents, again giving better acceptability in food applications for example. The present invention requires only sufficient acid to alter the charge on the anion exchange resin to result in release of the bound substances. It does not require excessive amounts of acids that may be recommended to clean ion exchange media on an empirical basis. In conventional ion exchange processes anion exchange resins are regenerated by the use of strong alkali.

While the method of the present invention operates preferentially at low temperatures for the adsorption stage and at high temperatures for elution (ie the regeneration step), the process will work, albeit at reduced efficiency, at almost any temperature of liquid water. It may actually be beneficial to move outside the preferred temperatures to fit process requirements and restrictions. For example, there may be advantages in operating the process at temperature extremes to prevent bacterial growth in the system used to operate the process. Such temperature extremes would be well known to persons skilled in the art.

The use of ion exchange resins in such separation processes has several advantages over the use of adsorbents. Such advantages include:

(i) ion exchange resins do not easily foul with large molecules because the charge on the resin covers most of the surface and only smaller molecules, such as the smaller peptides, will be able to bind in the spaces between the charges on the resin surface;

(ii) more ion exchange resins are permitted for food use applications by the Food and Drug Administration (USA) and their cost is generally subtantially lower than hydrophobic adsorbents;

(iii) by lowering the pH at high temperature, the ion exchange resins can readily be regenerated for further use;

(iv) ion exchange resins operated according to the protocol are relatively specific and will not remove all of the peptides from the hydrolysate mixture; and

(v) the ion exchangers can be operated in the neutral range and not in the hydroxide form eliminating base catalysed amino acid degradation which allows the recovery of the bound amino acids for potential later use.

Preferably the process is carried out by first washing the ion-exchanger according to manufacturer's recommendation and then washing in the presence of a salt (e.g. NaCl or the like). The ion-exchanger is then washed with buffer or water to achieve the desired pH for adsorption of amino acids and peptides, which is above the pK_a of the weak base ligands attached to the resin. The hydrolysate, dissolved in water at the appropriate equilibration pH, is then contacted with the resin in stirred batch mode or in column mode at a ratio of resin to hydrolysate sufficient to promote adsorption of the desired proportion of hydrophobic amino acids. Such ratios would be well readily determinable to a person skilled in this art. The resin is then drained and washed free of unbound hydrolysate product and the bound molecules are eluted with a regenerant, such as a buffer or acid, at a pH low enough to convert substantially all the weak base groups on the resin to the charged form. It is preferable that the adsorption step be carried out at low temperature to promote adsorption of the hydrophobic amino acids, and that the regeneration be carried out at high temperature to facilitate elution of the hydrophobic amino acids.

The resins can be washed with water and re-used without further equilibration if the process hydrolysate has significant buffering capacity to result in an equilibrium at a substantially neutral pH. If this is not the case, sufficient alkali must be added to achieve this.

EXAMPLES

To demonstrate the general principles involved in using the described invention, model bitter substances commonly associated with hydrolysates were used in the following examples. Such substances include L-Tryptophan, an aromatic amino acid with hydrophobic properties, and

its derivative L-Tryptophan methyl ester. In the latter the carboxylic acid group is derivatised to the non-charged methyl ester form, eliminating any charge effects of this group.

EXAMPLE 1 - L-TRYPTOPHAN UPTAKE ONTO A RANGE OF SYNTHETIC RESINS

The following experiment was performed to determine the extent of binding of a hydrophobic amino acid, at neutral pH, to a range of resins having different base matrix properties and varying strengths of ionic functionality. Synthetic polymeric resins based on polystyrene and acrylic are more hydrophobic than resins based on agarose, for example.

PROCEDURE

Binding of L-Tryptophan (L-Trp) to a number of synthetic weak and strong anion exchange resins, a synthetic hydrophobic adsorbent, and a hydrophilic weak anion exchange resin was tested as follows. Solutions of L-Trp (0.5% w/v) were prepared in 0.01M potassium phosphate, 0.5M NaCl buffer, pH 7.4, and mixed in batch mode with samples of each of the resins listed in Table 1 (10 mL/g wet resin) at 30°C for 16 hours.

The amount of non-bound amino acid was determined by OD measurement of the solutions at 280 nm, and from that, the amount of amino acid bound to each resin was calculated. The results are shown in Table 1 and Figure 1.

Table 1: Binding of L-Tryptophan to a series of resins to investigate the effects of charge type and base matrix properties on binding capacity.

Resin	Matrix type	Resin type	L-Tryptophan bound - mg/g wet resin
Diaion HP20	Styrene-DVB	Hydrophobic adsorbent	19.0
Pharmacia DEAE Sepharose	Agarose	Weak anion exchanger	0.2
Purolite A860	Acrylic	Strong anion exchanger	0.0
Amberlite IRA958	Acrylic-DVB	Strong anion exchanger	3.5
Purolite A107	Styrene-DVB	Weak anion exchanger	23.3
Diaion WA30	Styrene-DVB	Weak anion exchanger	12.1
Dowex MWA1	Styrene-DVB	Weak anion exchanger	10.6
Amberlite IRA93SP	Styrene-DVB	Weak anion exchanger	13.6
Purolite A103	Styrene-DVB	Weak anion exchanger	13.2
Purolite A100	Styrene-DVB	Weak anion exchanger	9.3
Purolite A105	Styrene-DVB	Weak anion exchanger	9.3
Relite A329	Styrene-DVB	Weak anion exchanger	14.5

DISCUSSION

Under these conditions, the hydrophobic adsorbent and the eight synthetic weak anion exchange resins bound the greatest amounts of L-Trp with varying efficiencies. To the

contrary, the two synthetic strong anion exchange resins and the hydrophilic agarose resin, bound very minimal amounts of L-Trp.

The results demonstrate the ability of the tested synthetic resins with hydrophobic base matrices and weak anion exchange functionalities to adsorb bitter hydrophobes to the same extent as hydrophobic adsorbents conventionally used for this purpose. Under the conditions of this Example, the strong anionic functional groups on the strong anion exchange resins interfere with the interaction.

EXAMPLE 2 - EFFECT OF pH ON ELUTION OF L-TRYPTOPHAN FROM AN ADSORBENT AND A WEAK ANION EXCHANGE RESIN

The purpose of this experiment was to determine optimal conditions for regeneration of weak anion exchange resins after they have been used to selectively remove bitter components from a process solution. To do this, the effect of pH was studied on the desorption of a hydrophobic amino acid from a weak anion exchange resin, and was compared to the effect of pH on the regeneration of an adsorbent.

PROCEDURE

Relite A329 was washed with 0.5M NaCl for 1 hour, rinsed with distilled water, and then equilibrated into 0.01M potassium phosphate buffer, pH 7.4. Diaion HP20 was equilibrated into 0.01M potassium phosphate buffer, pH 7.4. L-Tryptophan (L-Trp, 0.5% w/v in 0.01M potassium phosphate buffer, pH 7.4) was mixed with each resin in batch mode (10 mL L-Trp/g wet resin) at 30°C for 2 hours, and then filtered off. The resins were washed with the

equilibration buffer. Samples of both resins, bound with L-Trp, were suspended in water and then titrated to the following pH values using either 0.1M HCl or 0.1M NaOH as appropriate: pH 2, 4, 6, 9, 10. The amounts of L-Trp released from the resins were measured by OD of the solutions at 270 nm against a standard curve of L-Trp. The results are shown in Table 2 and Figure 2.

Table 2

Resin	Final pH	-Tryptophan released µg/g wet resin
Relite A329	1.99	0.5
Relite A329	4.06	10.3
Relite A329	6.03	7.5
Relite A329	6.87	5.5
Relite A329	9.08	4.5
Relite A329	10.1	3.6
Diaion HP20	1.99	8.4
Diaion HP20	3.99	8.3
Diaion HP20	6.00	7.3
Diaion HP20	7.47	7.8
Diaion HP20	9.04	7.
Diaion HP20	10.01	9

DISCUSSION

It should be noted that 100% recovery of L-Trp was not obtained from either of the two test resins under the conditions used, because in batch processes an equilibrium is formed between bound and free amino acid.

The drop in pH had a significant effect on the desorption of L-Trp from the weak anion exchange resin, with the optimal pH for regeneration of this resin being <pH 4. Desorption of L-Trp from the adsorbent resin, however, was independent of pH, and was less than the maximum amount released from the anion exchange resin.

The results show the advantage of using the ionic functionality of the anion exchange resins at low pH to regenerate the resin and release the adsorbed bitter components.

EXAMPLE 3 - EFFECT OF pH, TEMPERATURE AND SALT CONCENTRATION ON ELUTION OF L-TRYPTOPHAN FROM AN ADSORBENT AND A WEAK ANION EXCHANGE RESIN

To further optimise the conditons for regeneration of weak anion exchange resins, the following experiment was performed.

PROCEDURE

Relite A329 and Diaion HP20 were prepared as outlined in Example 2, and L-Trp was again bound to both resins under the conditions described. Samples of both resins bound with L-Trp, were suspended in water. Half of the resin samples were titrated to pH 4 using 0.1M HCl and the other half remained at pH 7.4. Resin samples at both pH conditions were mixed at 20°C, 50°C and 70°C for 1 hour. L-Trp released from the resins was removed by filtration and measured by OD at 270 nm. A sample of each resin was suspended in 0.1M NaCl, instead of water, and was mixed at pH 7.4 and 20°C. Again the released L-Trp was measured by OD at 270 nm. The results are shown in Table 3.

Table 3

Resin	pH	Temperature °C	NaCl added	L-Tryptophan released mg/g wet resin
Relite A329	4.0	20	No	12.9
Relite A329	4.0	50	No	17.1
Relite A329	4.0	70	No	20.7
Relite A329	7.4	20	No	7.8
Relite A329	7.4	50	No	11.2
Relite A329	7.4	70	No	15.6
Diaion HP20	4.0	20	No	9.9
Diaion HP20	4.0	50	No	13.0
Diaion HP20	4.0	70	No	17.2
Diaion HP20	7.4	20	No	10.4
Diaion HP20	7.4	50	No	13.1
Diaion HP20	7.4	70	No	19.0
Relite A329	7.4	20	Yes	8.8
Diaion HP20	7.4	20	Yes	9.8

DISCUSSION

Optimal release of L-Trp from the weak anion exchange resin was achieved with a combination of low pH (pH 4.0) and high temperature (70°C). Release of L-Trp from the adsorbent was significantly improved with increasing temperature, but unaffected by a drop in pH. As in Example 2, a shift in pH to ionise the weak anionic resin, provided a mechanism for release of the hydrophobic amino acid.

The presence of salt at a concentration typically present in milk had very little effect on the release of L-Trp from either resin.

EXAMPLE 4 - EFFECT OF TEMPERATURE AND pH ON THE ELUTION OF L-TRYPTOPHAN METHYL ESTER FROM RELITE A329

PROCEDURE

Relite A329 was treated in the following manner: washed with hot water (70°C), equilibrated in 0.5M NaOH, washed with cold distilled water (20°C), re-equilibrated into 0.5M NaCl and then given a final rinse with distilled water (20°C).

10mL volumes of L-Tryptophan methyl ester (L-TrpME, 0.5%w/v in 0.01M potassium phosphate buffer, pH 6.5) were added to 1 gram samples of washed resin and mixed for 1 hour at 20°C. The resin samples were filtered to remove unbound L-TrpME and washed with 0.01M potassium phosphate buffer, pH 6.5. A number of eluents were added to the resins and shaken for 1 hour at either 20°C or 80°C (see Table 4). L-TrpME released from the resin samples was removed by filtration and measured by OD at 270 nm against a standard curve of L-TrpME. The pHs of the final soltuions were measured. The results are shown in Table 4 and Figure 3.

TABLE 4 - THE EFFECT OF TEMPERATURE AND pH ON THE ELUTION OF L-TRYPTOPHAN METHYL ESTER FROM RELITE A329

Eluent	Temperature °C	Final pH	L-TrpME released mg/g wet resin
0.50M NaOH	20	13.4	5.1
0.05M NaOH	20	12.4	3.8

0.01M Phosphate, pH 6.5	20	7.1	9.3
Distilled water	20	6.6	10.4
0.05M HCl	20	2.9	15.9
0.50 HCl	20	<1.0	16.9
0.50M NaOH	80	13.3	9.4
0.05M NaOH	80	12.5	7.5
0.01M Phosphate, pH 6.5	80	6.6	17.4
Distilled Water	80	6.3	18.0
0.05M HCl	80	3.0	22.6
0.50M HCl	80	<1.0	23.8

DISCUSSION

The results show a dramatic increase in the release of L-TrpME from Relite A329 as the pH was lowered right down to pH 1. As in Example 3, the regenerating properties at low pH were enhanced at high temperature (80°C). Interestingly, strong alkali was ineffective at regenerating the resin.

EXAMPLE 5 - EFFECT OF RESIN POROSITY AND CHARGE DENSITY ON THE BINDING AND ELUTION OF L-TRYPTOPHAN METHYL ESTER

PROCEDURE

The resins used in this example were washed in 0.5M NaOH, rinsed with distilled water, and equilibrated into 0.5M NaCl. Prior to use, they were given a final rinse in distilled water. 0.5%w/v solutions of L-TrpME in 0.01M potassium phosphate buffer, pH 6.5, were added to resin samples (10mL/g wet resin) and mixed for 1 hour at 20°C. The resin samples were

filtered to remove unbound L-TrpME, and were washed with buffer.

Desorption of the amino acid from the resins was performed by mixing the resin samples with either 0.05M HCl or 0.01M potassium phosphate buffer, pH 6.5, for 1 hour at either 20°C or 80°C (See Table 5). The amounts of L-TrpME bound to, and released from, the resins were measured by OD at 270nm against a standard curve of L-TrpME. The results are shown in Table 5.

TABLE 5: BINDING AND ELUTION OF L-TRYPTOPHAN METHYL ESTER

Resin	L-TrpME bound mg/g wet resin	Eluent	Temperature of elution °C	L-TrpME released mg/g wet resin
Ionac AFP329	5.7	Phosphate	20	4.5
Ionac AFP329	5.8	Phosphate	80	4.1
Ionac AFP329	3.8	HCl	20	6.8
Ionac AFP329	4.7	HCl	80	6.2
Relite A329	19.8	Phosphate	20	12.4
Relite A329	22.5	Phosphate	80	15.6
Relite A329	20.4	HCl	20	20.2
Relite A329	20.5	HCl	80	20.7
Macronet MN100	32.9	Phosphate	20	0
Macronet MN100	33.5	Phosphate	80	0.5
Macronet MN100	35.1	HCl	20	17.5
Macronet MN100	35.5	HCl	80	16.9
Macronet MN150	33.7	Phosphate	20	0.1
Macronet MN150	30.6	Phosphate	80	0.1

Macronet MN150	32.0	HCl	20	23.5
Macronet MN150	31.7	HCl	80	21.1
Macronet MN200	36.1	Phosphate	20	0.4
Macronet MN200	35.9	Phosphate	80	0.1
Macronet MN200	33.3	HCl	20	12.4
Macronet MN200	35.1	HCl	80	13.1
Macronet MN250	30.5	Phosphate	20	0.5
Macronet MN250	29.2	Phosphate	80	0.2
Macronet MN250	No result	HCl	20	No result
Macronet MN250	31.9	HCl	80	15.3
Amberlite XAD16	30.8	Phosphate	20	2.9
Amberlite XAD16	30.2	Phosphate	80	3.6
Amberlite XAD16	27.2	HCl	20	16.2
Amberlite XAD16	29.5	HCl	80	15.8

DISCUSSION

This example illustrates two important effects: the importance of resin surface area for binding capacity, and the importance of charge density for desorption during regeneration of the resin.

The primary differences between resins Ionac AFP329 and Relite A329 are porosity and surface area. The former resin, with very low surface area, was unable to adsorb L-TrpME, and clearly demonstrated the requirement of the resin to have significant porosity for the process of the present invention. On the other hand, the Purolite Macronet resins have very high surface area and porosity, and thus displayed adsorptive properties for this amino acid, as good as, or even better than, the conventional adsorbent Amberlite XAD 16.

However, because the Macronet resins have either no surface charge (MN 200 and MN250) or only low densities of charged groups (MN100 and MN150, 0.4-0.6 mmoles/g dry resin), their regeneration properties at low pH were similar to the adsorbent resin, XAD 16. With Relite A329, 100% desorption of L-TrpME was achieved at low pH because of the much greater charge density of this resin (1.2 mmoles/g dry resin). Optimum desorption of the amino acid was achieved with low pH, high temperature and high density of charges on the resin.

EXAMPLE 6 - REMOVAL OF BITTER COMPONENTS FROM CASEIN AND WHEY HYDROLYSATES - COMPARISON OF 2 ADSORBENTS AND 1 WEAK ANION EXCHANGE RESIN

The aim of this experiment was to investigate whether the bitter components in the casein and whey hydrolysates, ie the hydrophobic amino acids, di and tri peptides, could be as effectively removed from the hydrolysates using a synthetic weak anion exchange resin, as compared to using conventional hydrophobic adsorbents.

Relite A329, Diaion HP20 and Diaion HP21 were washed in 0.5M NaOH, rinsed with distilled water, washed with 0.5M NaCl and finally rinsed with distilled water again. Casein hydrolysate and whey hydrolysate (10% w/v solutions in water) were mixed with the 3 resins (each at 2 mL hydrolysate/g wet resin, 10 mL/g and 50 mL/g) for 1 hour at 20°C. The non-bound material was removed from the resins by filtration and taste tested for elimination of bitter taste. OD measurements were performed at 214 nm and 280 nm to monitor levels of amino acids and peptides. The results are shown in Table 6 and Figure 4.

Table 6: COMPARISON OF ADSORBENTS AND A WEAK ANION EXCHANGE RESIN FOR THE REMOVAL OF BITTER COMPONENTS FROM CASEIN AND WHEY HYDROLYSATES

Resin	Solution applied	Wt of resin g wet	Volume 10%w/v hydrolysate	OD 280nm Treated hydrolysate	OD 214nm Treated hydrolysate	Taste
Diaion HP20	Casein hydrolysate	1.0	2	0.312	1.323	Acceptable
Diaion HP20	Casein hydrolysate	1.0	10	0.599	1.998	Acceptable
Diaion HP20	Casein hydrolysate	1.0	50	0.752	2.078	Bitter
Diaion HP20	Whey hydrolysate	1.0	2	0.217	1.268	Acceptable
Diaion HP20	Whey hydrolysate	1.0	10	0.476	1.903	Acceptable
Diaion HP20	Whey hydrolysate	1.0	50	0.563	2.072	Bitter
Relite A329	Casein hydrolysate	1.0	2	0.322	1.718	Acceptable
Relite A329	Casein hydrolysate	1.0	10	0.543	1.990	Acceptable

Relite A329	Casein hydrolysate	1.0	50	0.714	2.088	Bitter
Relite A329	Whey hydrolysate	1.0	2	0.257	1.679	Acceptable
Relite A329	Whey hydrolysate	1.0	10	0.488	2.007	Acceptable
Relite A329	Whey hydrolysate	1.0	50	0.593	2.036	Bitter
Diaion HP21	Casein hydrolysate	1.0	2	0.307	1.322	Acceptable
Diaion HP21	Casein hydrolysate	1.0	10	0.433	1.991	Acceptable
Diaion HP21	Casein hydrolysate	1.0	50	0.705	2.079	Bitter
Diaion HP21	Whey hydrolysate	1.0	2	0.181	1.128	Acceptable
Diaion HP21	Whey hydrolysate	1.0	10	0.231	1.961	Acceptable
Diaion HP21	Whey hydrolysate	1.0	50	0.413	2.045	Bitter
None	Casein hydrolysate		10	0.710	2.093	Bitter
None	Whey hydrolysate		10	0.638	2.070	Bitter

DISCUSSION

According to the taste test, the weak anion exchange resin (Relite A329) was as effective as the 2 adsorbents at reducing the bitter taste of whey and casein hydrolysates. However the capacity of all three resins to remove bitter components was exceeded at a ratio of 5g hydrolysate to 1g resin. By OD measurement at 280 nm, Relite A 329 was equally as effective as Diaion HP20, but slightly less effective than Diaion HP21, at removing aromatic amino acids from the hydrolysates.

EXAMPLE 7 - SPECIFICITY OF AN ADSORBENT AND A WEAK ANION EXCHANGE RESIN FOR AMINO ACIDS WITH DIFFERENT STRUCTURES

The purpose of the following experiment was to determine the specificity of a weak anion exchange resin and of an adsorbent resin for amino acids and peptides with different structures. The extent of binding of these compounds to the resins was determined at both neutral pH and low pH.

The amino acids tryptophan, phenylalanine and tyrosine contain aromatic rings, whilst valine, leucine and glycine contain aliphatic straight chains. All of these amino acids, and peptides containing these, except glycine, have hydrophobic properties and hence contribute to the bitter taste of hydrolysates.

PROCEDURE

Relite A329 was washed with 0.5M NaOH and then rinsed with distilled water. The pH of the resin was titrated to pH 7.0 using 0.5M HCl, and then rinsed again with distilled water. Amberlite XAD 16 was used as supplied.

A number of amino acids and peptides were mixed with these two resins using 10mL of 0.5% w/v solutions in 0.01M potassium phosphate, pH 6.5 buffer, and 1 gram samples of resin (Table 7). The samples were shaken at 20°C for 1 hour, and amounts of non-bound material were measured by OD at the optimum UV wavelength for each solution. The pH of the solutions were then lowered to pH 2 using approximately 10 mL of 0.1M HCl. Again the samples were shaken at 20°C for 1 hour. The amounts of non-bound material were re-

measured following the drop in pH. Spectral shifts in optimum UV wavelengths for each of the solutions was taken account of. The proportions of bound amino acids and peptides were calculated for both pH conditions, and are shown in Table 7 and in Figure 5.

Table 7 : Binding of amino acids and peptides with different structures

Resin	Solution applied	% Bound pH 6.5	% Bound pH <2
Relite A329	Trp-Leu	76	40
Relite A329	Val-Leu	1	0
Relite A329	Phe-Leu	63	4
Relite A329	Tyrosine	79	61
Relite A329	Leucine	0	0
Relite A329	Tryptophan	38	22
Relite A329	L-TrpME	57	14
Relite A329	Glycine	1	0
Amberlite XAD16	Trp-Leu	100	100
Amberlite XAD16	Val-Leu	4	5
Amberlite XAD16	Phe-Leu	77	49
Amberlite XAD16	Tyrosine	84	56
Amberlite XAD16	Leucine	5	15
Amberlite XAD16	Tryptophan	73	50
Amberlite XAD16	L-TrpME	74	73
Amberlite XAD16	Glycine	3	12

Trp	Tryptophan
Leu	Leucine
Val	Valine
Phe	Phenylalanine
TrpME	Tryptophan methyl ester

DISCUSSION

Amino acids and peptides containing only straight chain molecules did not bind to either of the two resins tested, showing that the base matrix of these resins is specific to compounds with aromatic properties. As in previous examples with L-Trp and L-Trp ME, the release of amino acids with aromatic structures, with drop in pH, was more dramatic with the weak anion exchange resin than with the adsorbent. This is interpreted as an effect of increased ionic functionality on the surface of the anion exchange resins induced by the addition of acid.

By way of explanation and for general clarity the following table (Table 8) describes the various adsorbents and resins that have been used in the previous Examples. The table details the name of the manufacturer, the resin type, the trade name of the resin, and the matrix type.

Table 8

Resin	Manufacturer	Resin Type	Matrix Type
Diaion HP20	Mitsubishi Kasei Corp	Hydrophobic adsorbent	Styrene DVB
Diaion HP21	Mitsubishi Kasei Corp	Hydrophobic adsorbent	Styrene DVB
Amberlite XAD16	Rohm and Haas	Hydrophobic adsorbent	Styrene DVB
Purolite A860	Purolite International	Strong anion exchanger	Acrylic
Amberlite IRA958	Rohm and Haas	Strong anion exchanger	Acrylic DVB
DEAE Sepharose CL-6B	Pharmacia	Weak anion exchanger	Agarose
Purolite A100	Purolite International	Weak anion exchanger	Styrene DVB

Purolite A103	Purolite International	Weak anion exchanger	Styrene DVB
Purolite A105	Purolite International	Weak anion exchanger	Styrene DVB
Purolite A107	Purolite International	Weak anion exchanger	Styrene DVB
Amberlite IRA93SP	Rohm and Haas	Weak anion exchanger	Styrene DVB
Diaion WA30	Mitsubishi Kasei Corp	Weak anion exchanger	Styrene DVB
Dowex MWA1	Dow Chemical Company	Weak anion exchanger	Styrene DVB
Relite A329	Sybron/Relite	Weak anion exchanger	Styrene DVB
Ionac AFP329	Sybron Chemicals Inc	Weak anion exchanger	Styrene DVB
Macronet MN-100	Purolite International	Weak anion exchanger	Styrene DVB
Macronet MN-150	Purolite International	Weak anion exchanger	Styrene DVB
Macronet MN-200	Purolite International	Hydrophobic adsorbent	Styrene DVB
Macronet MN-250	Purolite International	Hydrophobic adsorbent	Styrene DVB

DVB = divinyl benzene

The foregoing describes preferred forms of the invention and it is to be understood that the scope of the invention is not to be limited to the specific forms described. Modifications and variations as will be obvious to a person skilled in the art may be made to the forms of the invention as described without departing from the spirit or scope of the invention as defined in the attached claims.

1. A method for separating hydrophobic amino acids and peptides containing aromatic groups from protein hydrolysates using anion exchange media.

2. The method of claim 1 wherein the anion exchange media is an anion exchange resin.

3. The method of claim 1 or claim 2 wherein the anion exchange media has a hydrophobic base matrix with a weak base ion-exchange functionality.

4. The method of claim 3 wherein the weak base ion-exchange functionality has a pK_a or pK_a's in the pH range of substantially 2.0 to substantially 9.0.

5. The method of claim 3 wherein the weak base ion-exchange functionality has a pK_a or pK_a's in the pH range of substantially 2.0 to substantially 6.0.

6. The method of any one of claims 2 to 5 wherein the pH at which the anion exchange media is used to bind the amino acids and peptides is greater than the pH required to ionise the weak base functionality of the media.

7. The method of claim 6 wherein the pH at which the anion exchange media is used to bind the amino acids and the peptides is between substantially 4.0 and substantially 9.0.

8. The method of claim 6 wherein the pH is between substantially 6.5 and substantially 8.0.

9. The method of any one of the previous claims wherein the anion exchange media is regenerated for further use.

10. The method of claim 9 wherein the anion exchange media is regenerated by removing the bound amino acids and peptides from the anion exchange media using a low pH.

11. The method of claim 10 wherein the pH used to regenerate the anion exchange media is sufficiently low to create an ionised form of the media's weak base functionality.

12. The method of claim 10 wherein the pH used is between substantially 1.5 and substantially 6.0.

13. The method of claim 10 wherein the pH used is between substantially 1.5 and substantially 4.0.

14. The method of any one of claims 10 to 13 wherein the regeneration of the media uses a combination of low pH and high temperature.

15. The method of claim 14 wherein the temperature used is between substantially 45°C and substantially 100°C.

16. The method of claim 14 wherein the temperature used is between substantially 50°C and substantially 100°C.

17. The method of claim 14 wherein the temperature is between substantially 60°C and substantially 80°C.

18. A method of separating hydrophobic amino acids and peptides containing aromatic groups from a mixture of protein hydrolysates comprising:

(a) using an anion exchange resin at a substantially neutral pH, and thus in a substantially neutral form, to bind and remove said amino acids and peptides from said mixture or protein hydrolysates; and

(b) removing the bound amino acids and peptides from the anion exchange resin by lowering the pH to convert the substantially neutral resin to the substantially ionised form.

19. The method of claim 18 wherein the pH used in step (a) is between substantially 4.0 and substantially 9.0.

20. The method of claim 18 wherein the pH used in step (b) is between substantially 1.5 and substantially 6.0.

21. A method for the separation of hydrophobic amino acids and proteins containing aromatic groups from protein hydrolysates using a hydrophobic anion exchange resin with a weak base functionality comprising the steps of:

(a) equilibrating or regenerating the resin;

(b) adding the protein hydrolysate to the resin at a pH of between substantially 6.5 to substantially 8.0, at a temperature of between substantially 5°C and substantially 20°C, and at a concentration of between substantially 2% to substantially 20% w/v;

(c) mixing the hydrolysate and the resin;

(d) separating the non-bound product from the resin and rinsing with water; and

(e) regenerating the resin with hot water at a temperature of between substantially 60°C and substantially 80°C and at a pH of between substantially 1.5 and substantially 4.0.

22. The method of any one of the preceding claims wherein the protein hydrolysates are derived from animal products or plants.

23. The method of any one of the preceding claims wherein the protein hydrolysates are hydrolysates of casein, whey, or soy protein.

24. A method of separating hydrophobic amino acids and peptides containing aromatic groups from protein hydrolysate substantially as herein described with particular reference to the Examples and attached Figures.

25. Protein hydrolysate when formed by the method of any one of the previous claims.

26. A food product containing protein hydrolysate when formed by the method of any one of claims 1 to 20.

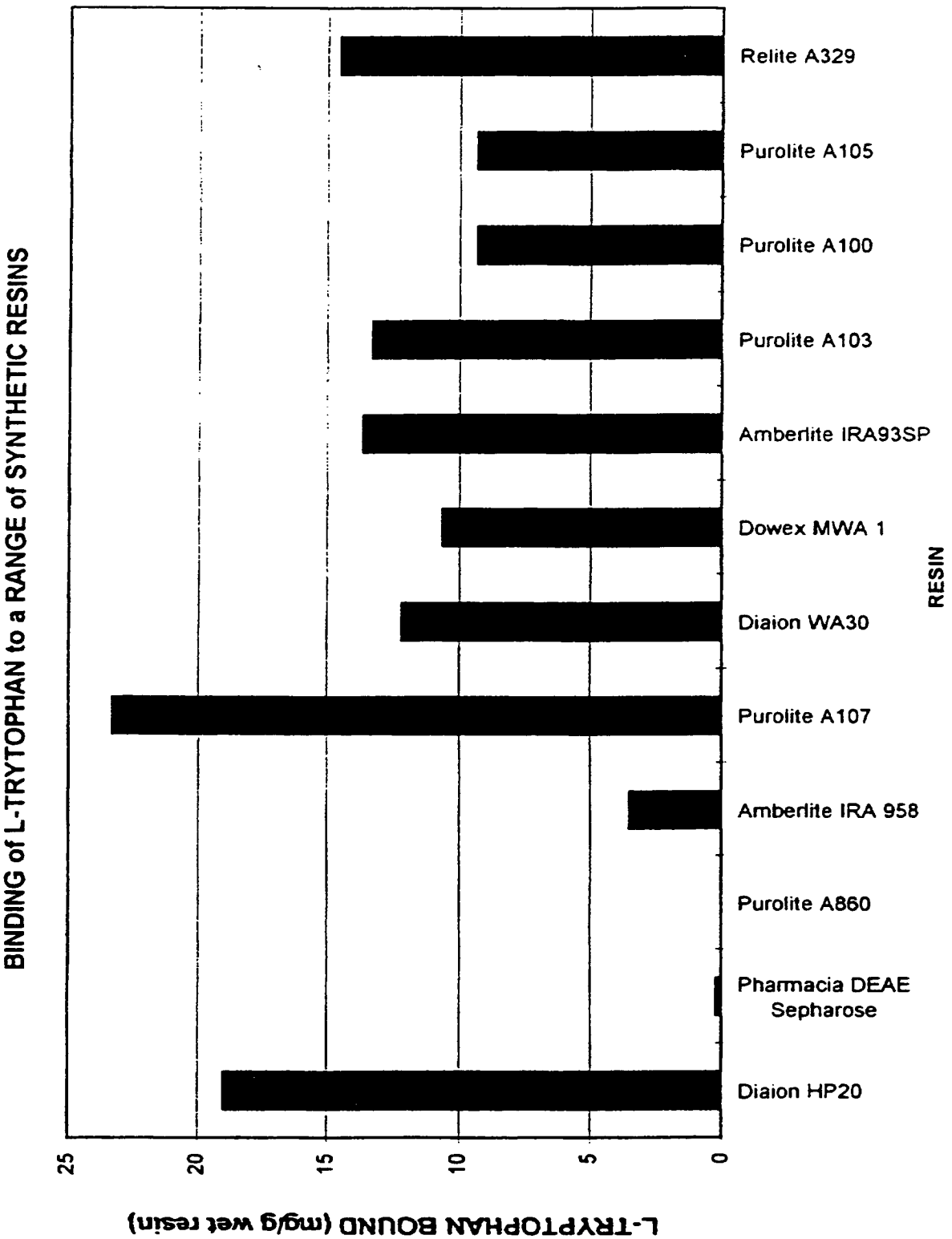


FIGURE 1

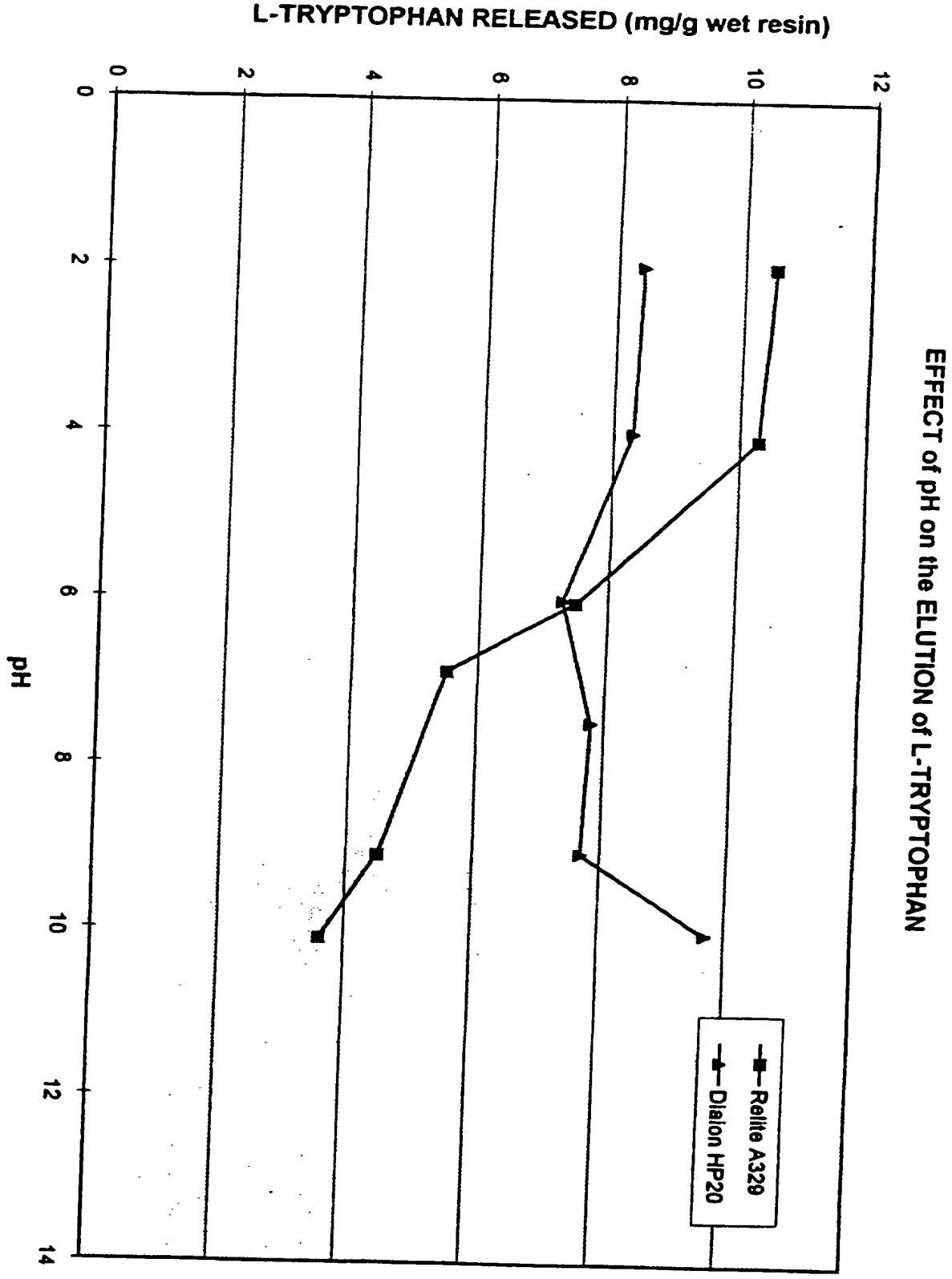
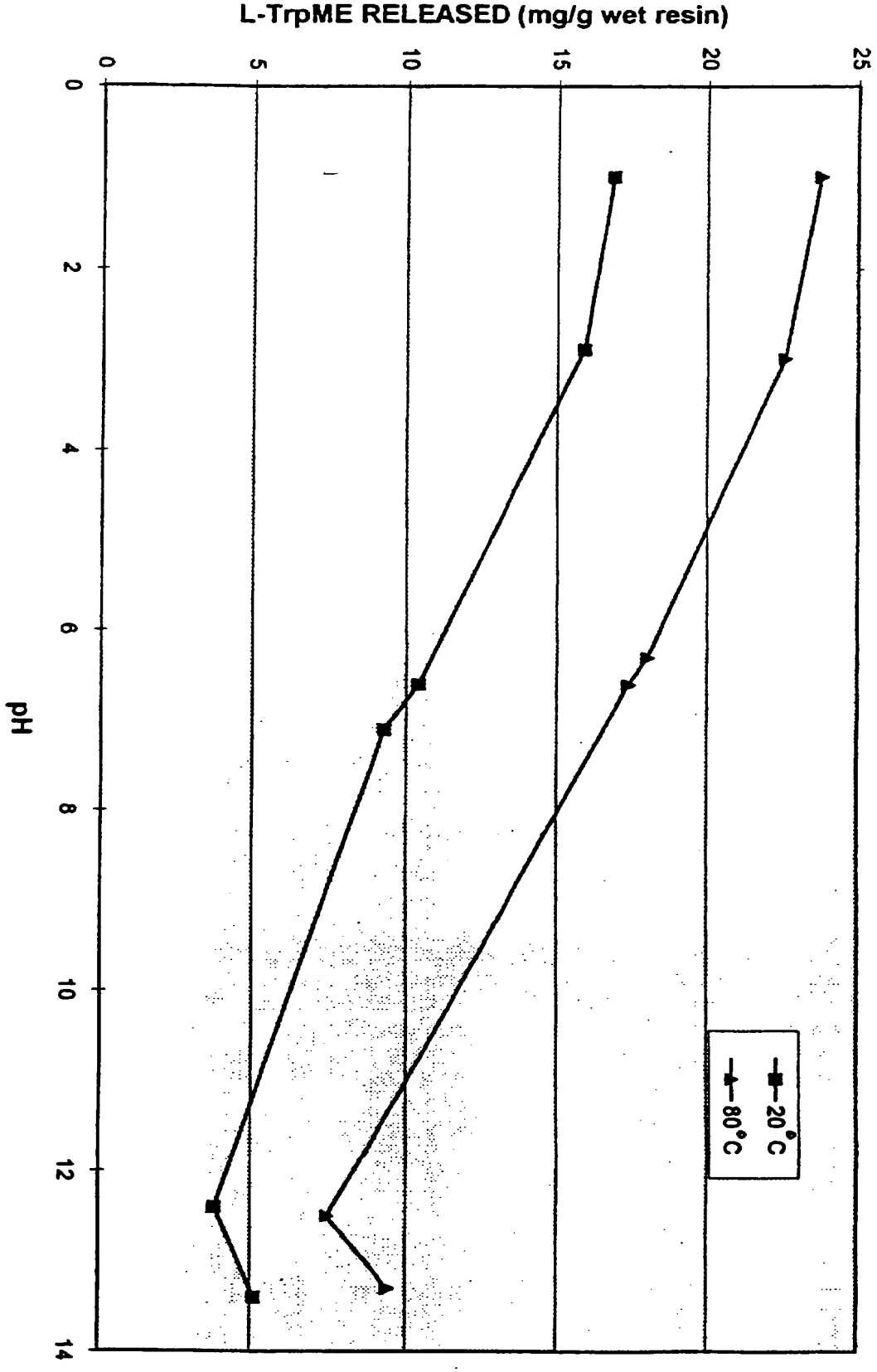


FIGURE 2



EFFECT OF pH AND TEMPERATURE ON THE RELEASE OF L-TRYPTOPHAN METHYL ESTER FROM RELITE A329

FIGURE 3

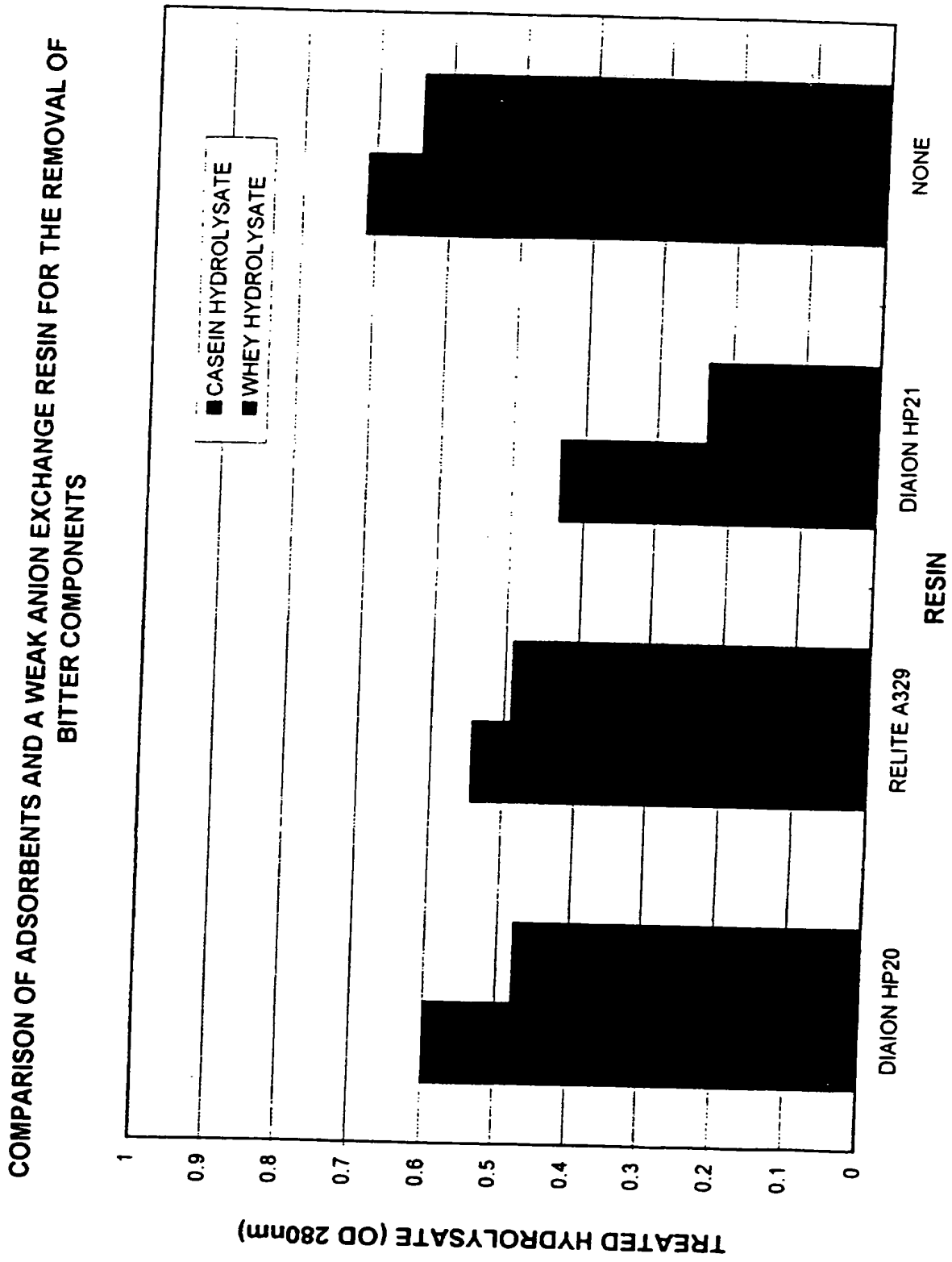
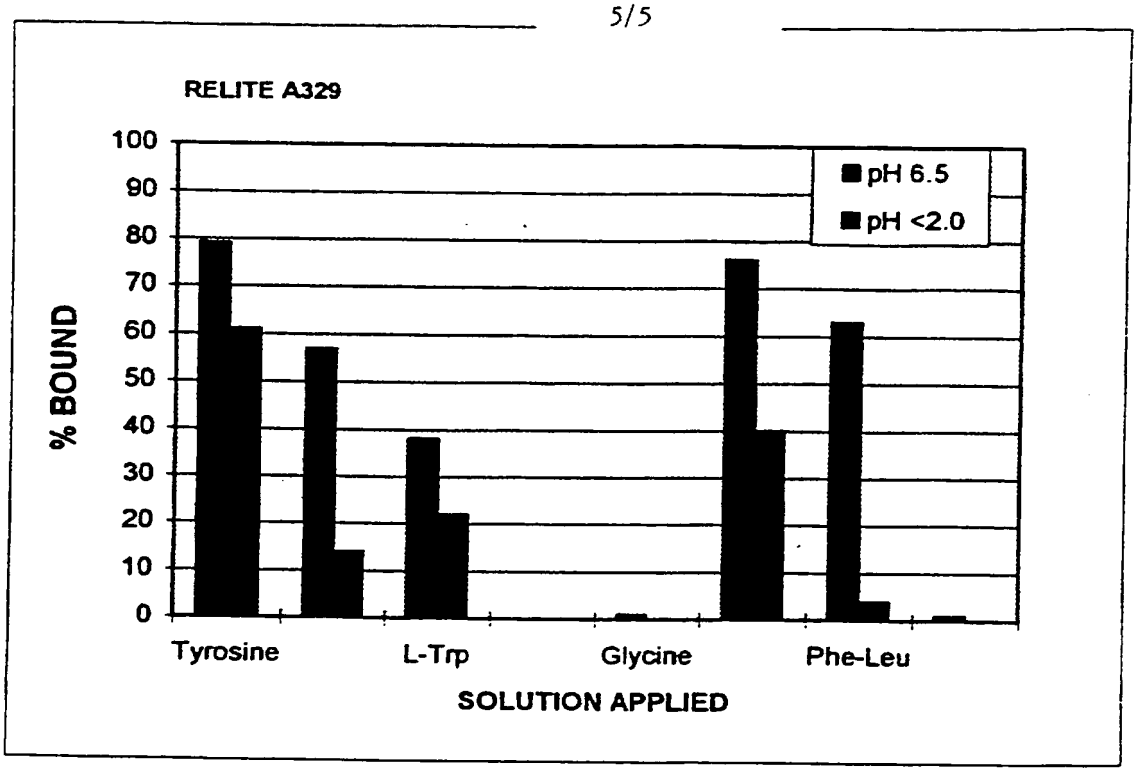


FIGURE 4



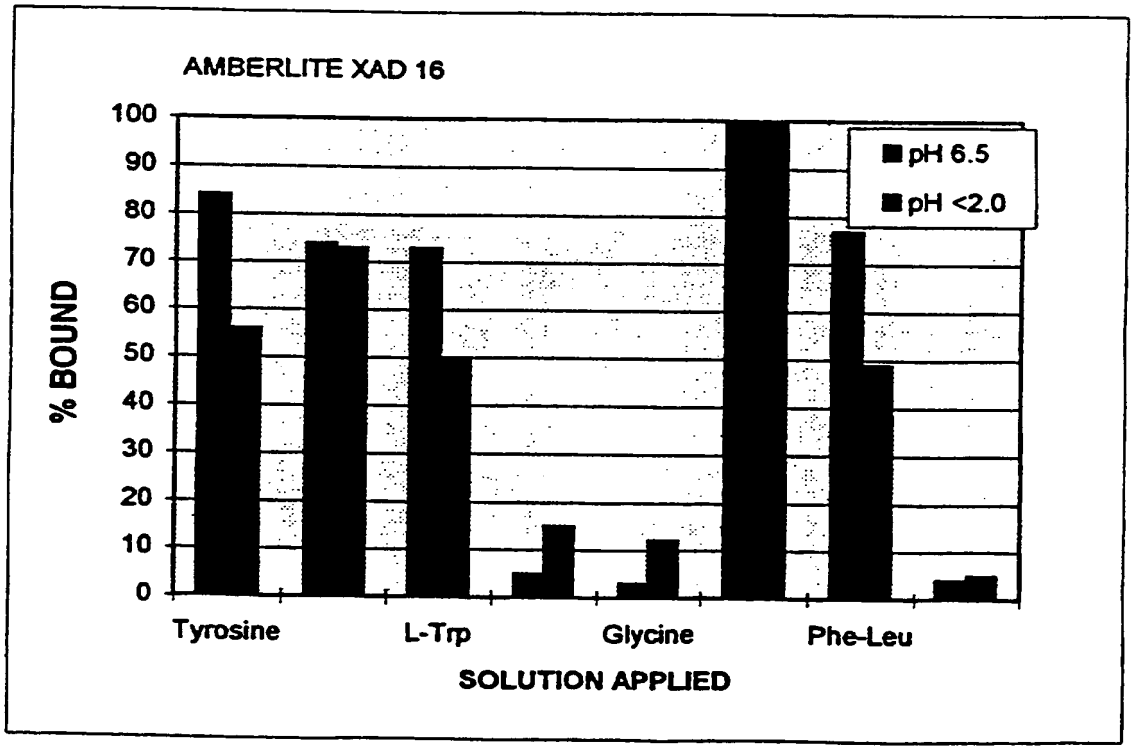


FIGURE 5

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/NZ 95/00107

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	4, 075, 195						
AU	76150/81	AT	12242	CA	1172978	DE	3107106
		DE	3169418	DK	4464/81	EP	49847
		ES	505959	FI	813108	IL	64011
		JP	57093916	NO	813407	NZ	198570
		US	4451455				
AU	55104/90	AT	73856	CA	1297050	DE	3585690
		DE	3688974	DK	3997/86	EP	216786
		EP	241539	EP	368367	ES	550260
		FI	855130	JP	7213291	JP	7213292
		JP	7213293	NZ	214711	WO	8603751
		US	5182196				

END OF ANNEX

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/NZ 95/00107

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C07K 1/18; A23J 3/08, 3/10, 3/16; A23L 1/015

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC[6] : C07K; C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT (WPAT; JAPIO): ANION##, ECHANGE#, ION##, C07C - 103/52/1C, PURIF:, SEPARAT:, EXTRACT:, HYDROPHOBIC, AROMATIC, AMINO ACID#, PEPTIDE#, PROTEIN#, ALANINE, ALA, ISOLEUGINE, ILE, LEUCINE, LEU, METHIONINE, MET, PHENYLALANINE, PHE, PROLINE, PRO, TRYPTOPHAN, TRP, VALINE, VAL
CHEMICAL ABSTRACTS (CA): (ANION? EXCHANGE?) / IT (ION? EXCHANGE?) / IT, SEPARAT?, PURIF?, ISOLAT?, REMOV?, EXTRACT?, 34/CC, 16/CC, 17/CC, 34/SX, 17/SX, 16/SX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	Derwent WPAT Online Abstract Accession No. 93-13419, JP 04341193-A (KANEBO LTD) 27 November 1992 Abstract	1-3, 18, 22, 23, 25, 26
X	Derwent WPAT Online Abstract Accession No. 92-280115, JP 04190797-A (FUJI OIL CO LTD) 9 July 1992 Abstract	1-3, 18, 22, 24, 25, 26

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 February 1996	5th March 1996

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INTERNATIONAL SEARCH REPORT

	International Application No. PCT/NZ 95/00107

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Chemical Abstracts, Volume 114, No. 25, issued 24 June 1991, Ohta, Hideaki et al, "Amino-nitrogen content and free amino acids of pineapple juice deacidified by means of ion exhange resins", page 655, abstract no. 246141j. Kinki Chugoku Nogyo Kenkyu 1990 (80) 59-63 (Japan)	
X	abstract	1-3, 18, 22
Y		4-17, 19-21, 23-26
	Journal of Chromatography, Vol 237, issued 1982 DIZDAROGLU, M et al "Separation of Peptides by High - Performance Liquid Chromatography on a weak anion - exchange bonded phase" pages 417-428	
X	whole document	1-3, 6, 7, 9, 10, 18, 19, 22, 25
Y	US, 4, 075, 195 (KRAFT, INC.) 21 February 1978, cited in the application whole document	4-17, 19-21, 23-26
X	AU-B-76150/81 (542795) (HOECHST A.G.) 22 April 1982 whole document	1-3, 6, 7, 18, 19, 22, 24, 25
X	AU-A-55104/90 (BIOGEN, INC.) 25 October 1990 whole document	1-3, 18, 19